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A Comparative Study of the Pathogenicity of *Fusarium circinatum* and other *Fusarium* Species in Polish Provenances of *P. sylvestris* L.

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Abstract: The fungal pathogen *Fusarium circinatum* is the causal agent of Pine Pitch Canker (PPC), a disease which seriously affects different species of pine in forests and nurseries worldwide. In Europe, the fungus affects pines in northern Spain and Portugal, and it has also been detected in France and Italy. Here, we report the findings of the first trial investigating the susceptibility of Polish provenances of Scots pine, *Pinus sylvestris* L., to infection by *F. circinatum.* In a greenhouse experiment, 16 Polish provenances of Scots pine were artificially inoculated with *F. circinatum* and with six other *Fusarium* species known to infect pine seedlings in nurseries. All pines proved highly susceptible to PPC and displayed different levels of susceptibility to the other *Fusarium* spp. tested. The findings obtained indicate the potentially strong threat of establishment of an invasive pathogen such as *F. circinatum* following unintentional introduction into Poland.

Keywords: *Pinus sylvestris*; pitch canker; *Fusarium circinatum*; damping-off; disease spread

1. Introduction

A substantial increase in the number of newly recognised invasive forest pathogens and of new destructive diseases has recently been noted in forest stands in Europe in the last few decades [1]. Among these, the fungal pathogen *Fusarium circinatum* Nirenberg & O'Donnell may pose a serious threat to the ecological and economic sustainability of forest ecosystems. In addition, climate change

is likely to predispose trees to attack from pathogens and will probably encourage the spread of pathogens into new areas [2,3].

The ascomycete fungus *Fusarium circinatum* infects a wide range of pine *Pinus* species and can cause pitch pine canker (PPC). Although *Pinus radiata* D.Don seems to be the most susceptible species [4], up to 60 pine species have been reported to be susceptible to PPC, including Mediterranean species such as Aleppo pine (*P. halepensis* Miller) and Maritime pine (*P. pinaster* Aiton), as well as European species such as Scots pine (*P. sylvestris* L.) and also various North American and Asian species planted in Europe, such as Lodgepole pine (*P. contorta* Douglas), white pine (*P. strobus* L.), Japanese red pine (*P. densiflora* Siebold & Zucc.) and Japanese black pine (*P. thunbergii* Parl.) [5,6]. The pathogen is widely distributed and causes dieback and death of susceptible pine species. *Fusarium circinatum* was first found in North America, and to date the disease has been reported in Central and South America, South Africa, Asia, USA, Mexico, Haiti, Japan, Chile [7] and, more recently, in Europe. Nowadays, the disease is present in forests in Spain [8] and Portugal [9] and it has also been reported in parts of France and Italy [10,11], although it has probably subsequently eradicated from both of the latter countries [12,13]. The disease threatens the sustainability of pine forests in Spain, Portugal, France, Italy and Greece, among other countries. These areas are at risk, as confirmed by climatic data and host distribution [2,3]. However, other European countries are also at risk of infection [4–6]. An increase in imports of live plants and other material has led to a high risk of the pathogen being introduced through infested goods. However, considering the high level of susceptibility of young pines to the disease, and the ease of infection via seeds or seedlings, the threat of *F. circinatum* to European nurseries is particularly serious.

Scots pine (*Pinus sylvestris*) is a common and economically important timber species in Europe. The widespread distribution of an alien invasive pathogen such as *Fusarium circinatum* thus poses a serious threat to the biodiversity of forest ecosystems, especially Scots pine stands. In 2007, the European Commission adopted provisional emergency measures to prevent the introduction and spread of *Gibberella circinata* Nirenberg and O'Donnell (the sexual stage of the pitch canker pathogen) within the Community (Commission Decision 2007/433/EC of 18 June 2007) [6]. The presence of *F. circinatum* in forest plantations and nurseries has led to large reductions in crop yields as well as loss of revenue due to the high costs of monitoring and control of the pathogen and to bans on planting susceptible species (*Pinus* spp. and *Pseudotsuga menziesii* (Mirb.) Franco) in infected areas in Spain and on the export of timber and other products (Spanish Royal Decree 637/2006 and 65/2010). Nurseries in which plants were infected by *F. circinatum* were typically the first point of entry of the disease and still constitute a reservoir for the transmission of the PPC to forest plantations. Moreover, various *Fusarium* spp. affect the viability of seeds and young pine plants, causing damping-off disease and resulting in losses due to non-viable seeds and weakening or death of seedlings. *Fusarium* spp. are reported to play an important role in the development of damping-off disease in plantations and forest nurseries, mainly when seedlings are grown in containers with peat moss, due to the acidic nature of this substrate [14]. *Fusarium oxysporum* is a soil-borne fungus species that can act as a saprophyte as well as an aggressive root and seed pathogen in a wide variety of hosts including Scots pine seedlings and many economically important crops [15–17]. In Poland, most forest tree nurseries have experienced serious problems with damping-off rot caused by *Rhizoctonia* and *Fusarium* spp. [18,19]. Some seed-borne *Fusarium* spp. have been recognized to be aggressive pathogens of pine seedlings and crops. The establishment of pine plantations on former farmland in Poland may lead to infection of pine seeds and seedlings by different *Fusarium* spp. remaining in the soil.

The aim of this research was to test the pathogenicity of *F. circinatum* and other *Fusarium* species in order to evaluate the threat posed by these fungi, which may potentially affect seedlings of Polish provenances of *P. sylvestris*.

With this aim, we compared the development of pitch canker symptoms in Polish provenances of Scots pine seedlings to establish whether the susceptibility or tolerance of plants to *F. circinatum* is

related to their geographical origin. Moreover, we also assessed the potential effects of six isolates of other *Fusarium* spp., including species typically affecting agricultural crops.

2. Materials and Methods

2.1. Fungal Isolates

An inoculum of *Fusarium circinatum* was produced by growing the fungus on potato dextrose agar (PDA) for seven days in Petri dishes at 25 °C under laboratory conditions. In addition, isolates of *Fusarium poae, F. oxysporum, F. graminearum* (A and D isolates), *F. tricinctum* and *F. culmorum* of Polish origin were selected for comparison of virulence (Table 1).

Table 1. Origin of isolates of *Fusarium* species used in the study.

Species	Code	Host	Location, Country	Latitude	Longitude
Fusarium circinatum [20]	FcCa6	*Pinus radiata*	Comillas, Cantabria, Spain	43°20′16.2″ N	4°18′17.1″ W
Fusarium poae	A	Winter wheat ears	Bohemia NE Poland *	54°09′45″ N	9°00′40″ E
Fusarium graminearum	A	Winter wheat grain	Ozon NE Poland *	54°09′45″ N	9°00′40″ E
Fusarium tricinctum	A	Winter wheat grain	Julius NE Poland *	54°09′45″ N	9°00′40″ E
Fusarium oxysporum	D	Winter wheat	Julius NE Poland *	54°09′45″ N	9°00′40″ E
Fusarium culmorum	D	Winter wheat	Julius NE Poland *	54°09′45″ N	9°00′40″ E
Fusarium graminearum	D	Winter wheat	Julius NE Poland *	54°09′45″ N	9°00′40″ E

* NE—North Eastern.

For the in vitro virulence test, we followed the method of James et al. (1997), as briefly described below [21].

2.2. In Vitro Virulence Test

Stratified seeds of three different Polish provenances of *P. sylvestris* (Klosnowo, Lasowice and Dębno, Table 2) were placed inside a mesh bag and soaked in tap water. The seeds were germinated on moistened, sterile Whatman No. 3 filter paper in sterile Petri dishes and incubated at about 22 °C. After 15 days, the pine seedlings were transferred to Petri dishes containing the fungal inocula. Thus, for each fungal isolate, 12 Petri dishes each with two of the seedlings were prepared (i.e., 24 replicates). The Petri dishes were incubated at approximately 23 °C under artificial lighting (160 Watts per m^2), and sterile water was added as required. Disease symptoms (root rot, damping-off, and disease free, respectively) were monitored over a 21-day period.

Table 2. Origin of Polish provenances of *Pinus sylvestris* used in the study.

Scots Pine Provenance *	Latitude	Longitude
Tuszyma	50°11′4.342″ N	21°29′53.38″ E
Wejherowo	54°36′6.144″ N	18°13′46.42″ E
Kluczbork	51°58′23.639″ N	18°12′32.983″ E
Dojlidy	53°9′5″ N	23°8′48.094″ E
Kutno	52°18′9.413″ N	19°17′40.454″ E
Kwidzyn	53°44′12.331″ N	18°55′45.452″ E
Tuchola	53°40′9.624″ N	17°54′31.928″ E
Oborniki Śląskie	51°17′53.093″ N	16°54′41.685″ E
Lidzbark	53°15′3.828″ N	19°46′13.794″ E
Karczma Borowa	51°51′4.417″ N	16°36′54.071″ E
Lubsko	51°47′22.074″ N	14°58′30.576″ E
Czaplinek	53°33′44.406″ N	16°13′37.974″ E
Jablonna	52°23′1.454″ N	20°56′1.816″ E
Klosnowo	53°47′60″ N	17°37′60″ E
Lasowice	50°54′16.443″ N	18°15′19.283″ E
Debno	52°37′14.16″ N	14°37′30.632″ E

* Forest District.

After 21 days, all germlings were harvested and examined for disease symptoms before being re-isolating the fungi onto malt extract agar (MEA). Seedling survival was scored as follows: 1 point was awarded for each day the seedlings survived (maximum 15 points); another 1, 2 or 5 points were awarded depending on the type of disease (root rot, damping-off, and disease free, respectively); and 5 addition points were awarded when the root doubled or more in size. The scores ranged from 5–25, with higher values reflecting lower virulence of the isolate. The scores were converted to a reciprocal score of 0–100, with virulence ratings of 0 indicating no fungal infection, and 100 indicating that the seedlings died within three days [21,22].

Based on the findings of previous studies [19,21,22], virulence scores >60 indicated moderate-high virulence, scores between 40 and 59 indicated low virulence, and scores below 39 indicated lack of virulence.

2.3. Greenhouse Pathogenicity Tests

Preliminary assessment of the pathogenicity of *F. circinatum* and other *Fusarium* species for Polish provenances of pine seedlings was carried out at the Research Centre of Quarantine, Invasive and Genetically Modified Organisms, Institute of Plant Protection—NRI (Poznan, Poland). The *F. circinatum* and other *Fusarium* isolates were cultivated on PDA at 24.5 °C for seven days (Table 1).

Two-month-old seedlings of 12 Polish provenances (37–42 seedlings per provenance) and 72 one-year-old seedlings of another Polish provenances of *Pinus sylvestris* (Jablonna) were used in preliminary pathogenicity tests (Table 2).

Inoculums of *F. circinatum* were added to pots in which individual two-month-old pine seedlings were growing, by pouring spore suspensions (ca. 1×10^6 CFU/mL water) on to the soil (approximately 1×10^6 CFU per seedling).

For each of the 7 *Fusarium* species tested, the one-year-old pine seedlings were inoculated by making a wound on the stem with the aid of a scalpel and pipetted 10 µL of *Fusarium* spore suspension (1 million spores mL^{-1}) onto the wound [20]. The wound was then covered with a strip of Parafilm to prevent desiccation. Control seedlings were inoculated in the same way, with *sterilized water*, and the wounds were also covered with a strip of Parafilm.

The morphological condition of all pine seedlings was first evaluated on a weekly, and then a monthly basis as visually healthy, weakened or dead plants subject to typical symptoms of pitch canker or damping-off. Disease susceptibility was estimated at 60 and 120 days post-inoculation (dpi).

Samples of seedlings, 5 mm length segments, were placed in Eppendorf tubes. Whole samples of the two-month-old seedlings were analyzed, while two segments of length 5 mm were cut from the stem of each one-year-old seedling. The samples were homogenized by cutting them into pieces with scissors and shaking the pieces in tubes with two glass beads. Subsamples (500–700 uL) of all homogenized samples were transferred to 2 mL screw cap tubes. The tubes were then placed overnight in a freeze-drier with the lids loosely screwed on (covered with Parafilm to keep them in place). All samples were analyzed using conventional PCR tests for detection/reisolation of *F. circinatum* with species-specific primers (CIR1 and CIR4 described below).

2.4. DNA Extraction, Purification and PCR Amplification

Fresh or frozen samples were ground to a fine powder with liquid nitrogen in a mortar and pestle before DNA extraction. Ground material (500 to 700 mg) was transferred to a sterile microfuge tube. The DNA was extracted and purified using the NucleoSpin® Plant II Midi kit (MACHEREY-NAGEL product, Düren, Germany). The DNA of the samples was then quantified along with standard reference samples in a NanoDrop ND-1000 spectrophotometer (Wilmington, DE, USA). The DNA in individual samples was diluted to 5–10 ng/µL.

The diluted DNA samples were analyzed by conventional PCR with species-specific primers to detect the presence/absence of *F. circinatum*. The specific forward primers CIRC1A (TCG ATG TGT CGT CTC TGG AC) and reverse primers CIRC4A (CGA TCC TCA AAT CGA CCA AGA) were used to

amplify the IGS rDNA region [23]. The PCR reaction mixture included 1× PCR buffer supplied with the DNA polymerase, 0.25 mM each dNTP, 2 mM $MgCl_2$, 0.5 µM of each CIRC1A and CIRC4A primer, 0.05 U/µL DNA polymerase and 6.0 µL of DNA.

The PCR reaction was carried out in a thermocycler Veriti 96 Thermal Cycler (Life Technologies™, Life Technologies™, Camarillo, CA, USA) and involved initial denaturation at 95 °C for 3 min, followed by 40 cycles consisting of denaturation at 95 °C for 30 s, annealing at 64 °C for 55 s and elongation at 72 °C for 50 s. The final elongation step was carried out at 72 °C for 12 min. The PCR products were visualized by electrophoresis in a 1% agarose gel.

For identification of the *Fusarium* species, the internal transcribed spacer (ITS) region was analyzed. In addition, the β-tubulin gene and the partial elongation factor 1-alpha (EF1-a) gene were amplified and sequenced for fungi from the genera. The β-tubulin gene was amplified using Bt2a and Bt2b primers [24] and the EF1-a gene was amplified using the EF1F and EF2R primers, following the protocols reported by Kristensen et al. (2005) for the identification of *Fusarium* spp. [25].

For PCR amplification of the ITS regions of fungal ribosomal RNA (ITS rRNA), the primers ITS1F [26] and ITS4 [27] were used. Each PCR reaction contained 200 µM deoxyribonucleotide triphosphates, 0.2 µM of each primer, 0.03 U/µL Thermo Green Taq polymerase with reaction buffer, and 2.75 mM $MgCl_2$ (final concentration). The initial denaturation step at 95 °C for 5 min was followed by 35 amplification cycles consisting of denaturation at 95 °C for 30 s, annealing at 55 °C for 30 s, and extension at 72 °C for 30 s. The thermal cycling was ended by a final extension step at 72 °C for 7 min. PCR products were size separated on 1% agarose gels and visualized under UV light. PCR conditions for EF1-a amplification were the same as those for ITS except for the annealing temperature, which was 60 °C. The thermal cycling condition for the β-tubulin gene was 2 min at 95 °C followed by 35 cycles of 95 °C for 30 s, 58 °C for 45 s, and 72 °C for 45 s, with a final extension of 72 °C for 5 min.

The raw sequence data were analyzed using the SeqMan Pro version 10.0 software included in the DNASTAR package (DNASTAR, Madison, WI, USA). The identity of the ITS rRNA sequences was determined by comparison with GenBank databases [28]. The following criteria were used for the identification: sequence coverage >80%; similarity to taxon level 98%–100%; similarity to genus level 92%–97%.

2.5. Statistical Analysis

All data were analyzed using Kolmogorov–Smirnov test to check for adherence to the normal distribution and Bartlett's test to check the homogeneity of variance. Data on lesion length of Scots pine seedlings inoculated with *F. circinatum* or other *Fusarium* species were subjected to analysis of variance (ANOVA) by using the general linear model (GLM) module in Statistica software STATISTICA® 7.0 (StatSoft, Inc., Tulsa, OK, USA). When the ANOVA indicated significant treatment effects ($p < 0.05$), a post hoc HSD Tukey test was used to compare the means. For all fungal isolates, Chi-square tests were used to determine any differences in plant mortality. Means were compared using a post hoc HSD Tukey test with a significance level of $p < 0.05$. Confidence intervals were determined to distinguish between treatments representing different seedling provenances and *Fusarium* species. Following the ANOVA, significant differences between treatments were further evaluated by a post hoc HSD Tukey test. Survival analysis was performed to test the mortality until the end of the experiment by using the nonparametric Kaplan-Meier estimator with the "Survival" package [29] implemented in an R software environment (R Foundation for Statistical Computing, Vienna, Austria).

3. Results

3.1. Effect of Fusarium Species on Pine Seedling

The growth of pine seedlings inoculated with different species of *Fusarium* varied considerably (Table 3). *Fusarium circinatum* killed almost all the pine seedlings in this assay, but all other isolates of *Fusarium* tested, i.e., *F. poae*, *F. graminearum* (A and D), *F. tricinctum* and *F. culmorum*, had little impact

on seedling health (Table 3). Only *F. oxysporum* strongly affected the plant development, resulting in mortality of 62.5%–66.7% of the seedlings by day 21.

Both the mortality rate and root growth inhibition were almost equal in all provenances of Scots pine seedlings tested (F = 2.6356, p = 0.104 and F = 1.8089, p = 0.5291). *F. oxysporum* caused the highest mortality rates (62.5%–66.7%, F = 9.3473, p = 0.001053)), while *F. poae, F. graminearum* (A and D) and for *F. culmorum* caused low morality rates (0%–4.2%). *F. tricinctum* caused intermediate mortality rates of 12.5%–20.8%. *F. oxysporum* also significantly reduced plant growth (32% of plants weakened). No dead or weakened seedlings were observed in the control treatments. In other variants the mortality did not exceed 5% and the number of weakened seedlings varied from 0% to 4%, except for *F. tricinctum* (6.1%–8.3% of seedlings weakened and mortality rate, 12.7%–20.5%). Root growth was strongly inhibited by *F. oxysporum* (79.4%–88.7%) causing extensive vascular discoloration of the root tissue and affecting seedling viability (p = 0.03 for root growth inhibition and p = 0.00124 for mortality). *F. tricinctum* also inhibited root growth (58.2%–64.5%, p-value = 0.0152), while *Fusarium poae*, *F. graminearum* (A and D) and *F. culmorum* caused low levels of root growth inhibition of respectively 14.4%–16.2%, 17.8%–19.8%, 18.5%–20.5% and 18.5%–20.4% (Table 3), The virulence scores for *Fusarium poae, F. graminearum* (A and D) *F. tricinctum* and *F. culmorum* were on average, 11.3, 13.3 and 11.7, 11.3 and 33.7 (data not shown, rate from 0 to 100) indicating that these species are non-pathogenic. Although *F. culmorum* was awarded significantly higher scores (p-value = 0.014), this isolate may also be included in the group of isolates displaying low virulence for pine seedlings. Only *F. oxysporum* was awarded high scores for all three provenances of seedlings tested (average score 52) and can be considered moderately-highly virulent. Among tested Fusarium spp., *Fusarium circinatum* is the highest level mortality for pine seedlings and is the most virulent pathogen.

3.2. Greenhouse Pathogenicity Tests

Artificial inoculation of seedlings with *F. circinatum* led to the formation of necrotic lesions, resin flow of and mortality of 90% of one-year-old seedlings of one of the Scots pine provenances from Poland (Jablonna) (Table 4) during the fourth month of the assay.

Table 3. Effects of different *Fusarium* isolates on *Pinus sylvestris* growth and root development.

Isolate	Klosnowo				Lasowice				Dębno			
	Root Inhibition, %	Mortality, %	Points	Virulence Score	Root Inhibition, %	Mortality, %	Points	Virulence Score	Root Inhibition, %	Mortality, %	Points	Virulence Score
Fusarium poae A	14.4 ± 2.8^{a}	4.2 ± 0.07^{a}	22.54 ± 0.4^{a}	12.5	16.2 ± 0.9^{a}	0^{d}	22.13 ± 0.2^{a}	14.5	15.7 ± 0.2^{a}	4.2 ± 0.1^{a}	22.17 ± 1.7^{a}	14
Fusarium graminearum A	18.9 ± 1.1^{b}	4.2 ± 0.03^{a}	21.75 ± 2.1^{a}	16	19.8 ± 1.1^{b}	4.2 ± 0.1^{a}	21.33 ± 0.9^{a}	18.5	17.8 ± 1.8^{b}	4.2 ± 0.1^{a}	21.96 ± 1.6^{a}	15
Fusarium tricinctum A	64.5 ± 2.6^{c}	16.7 ± 2.7^{b}	16.87 ± 0.9^{b}	40.5	59.8 ± 3.4^{c}	12.5 ± 1^{b}	16 ± 1.1^{b}	44.5	58.2 ± 3.5^{c}	20.8 ± 1.4^{b}	16.79 ± 1.3^{b}	41
Fusarium oxysporum D	88.7 ± 6.8^{d}	62.5 ± 12.1^{c}	11.7 ± 0.72^{c}	66.5	80.4 ± 7.8^{d}	66.7 ± 9^{c}	12.2 ± 1.1^{c}	64	79.4 ± 6.7^{d}	66.7 ± 5.1^{c}	12.87 ± 0.9^{c}	60.5
Fusarium culmorum D	18.5 ± 1.3^{b}	4.2 ± 0.04^{a}	21.62 ± 2.0^{a}	17	20.5 ± 1.91^{b}	0^{d}	21.8 ± 1.7^{a}	16	20.4 ± 1.4^{b}	0^{d}	21.8 ± 1.4^{a}	16
Fusarium graminearum D	19.6 ± 2.1^{b}	0^{d}	22.41 ± 2.1^{a}	13	18.5 ± 1.5^{b}	0^{d}	22.17 ± 1.8^{a}	14	20.5 ± 2.3^{b}	0^{d}	21.79 ± 1.7^{a}	16
Fusarium circinaum	98.8 ± 1.1^{e}	100^{e}	1.62 ± 0.02^{e}	99	96.7 ± 2.1^{e}	100^{e}	1.62 ± 0.02^{e}	99	98.8 ± 1.1^{e}	97.1 ± 2.1^{e}	1.62 ± 0.02^{e}	98
Control	1.7 ± 0.1^{e}	0^{d}	24.8 ± 1.7^{a}	1	1 ± 0.1^{e}	0^{d}	24.9 ± 1.7^{a}	0.5	1.2 ± 0.1^{e}	0^{d}	24.9 ± 1.7^{a}	0.5

With columns, values followed by different letters are significantly different (HSD Tukey post hoc test, $\alpha = 0.05$).

Table 4. Level of mortality and lesion length (mm) in one-year-old *P. sylvestris* seedlings inoculated with different species of *Fusarium*.

	F. circinatum	*F. poae A*	*F. graminearum A*	*F. tricinctum A*	*F. oxysporum D*	*F. culmorum D*	*F. graminearum D*	Control
Mortality, %	90	0	0	0	0	0	0	0
Weakened plants, %	5	0	0	0	5	0	0	0
Lesion length, mm^2	10.4 ± 2.1^{b}	2.15 ± 0.08^{a}	2.27 ± 0.21^{a}	2.35 ± 0.64^{a}	2.45 ± 0.37^{a}	1.9 ± 0.62^{a}	2.36 ± 0.5^{a}	1.3 ± 0.09^{a}
Range, mm	3.7–14.2	1–2.2	1.2–4.2	1.4–3.6	1.7–3.8	1–2.2	1.1–2.7	1–1.4

With columns, variables followed by the same letter are not significantly different ($p = 0.05$: post hoc HSD Tukey test).

During the first two months of the trial, small necrotic lesions of 1.6–2.8 mm were observed in 30% of the seedlings, with no clear symptoms of pitch canker, while necrotic lesions 5.3–6.7 mm in length were observed in 40% of seedlings, which also showed typical symptoms of disease such as basal needle dieback, wilting and side shoot dieback. The longest necrotic lesions (8.4–8.7 mm) were observed in 30% of dead seedlings two months after inoculation. The lesions were generally covered with resin and extended vertically in both directions from the point of inoculation. Dead and declining seedlings showed symptoms of stem necrosis, discoloration and loss of needles. No dieback or decline was observed in the control seedlings.

Inoculation of seedlings with the other six *Fusarium* spp. commonly led to the formation of small necrotic wounds, which did not differ significantly in size from those observed in the control seedlings (Table 4). Only *F. oxysporum* demonstrated very low virulence causing discoloration of needles around the inoculation point; the other *Fusarium* species did not cause any symptoms of weakening, shoot decline or seedling mortality (Table 4). Moreover, there were no significant differences in the size of the lesion or seedling condition in relation to the six *Fusarium* spp. One-year-old seedlings inoculated with the *Fusarium* species other than *F. circinatum* showed no symptoms of weakening or damping off ($p < 0.01$).

The inoculated *Fusarium* spp. (including *F. circinatum*) were successfully re-isolated from 100% of the plants. The identity of samples was 99%–100% consistent with *F. circinatum* strain FCC4880 28S-18S, GenBank: KC147556.1. All re-isolated fungal species and wood samples from the inoculated and control seedlings used for direct amplification and sequencing of the fungal IGS rDNA region yielded successful amplification. The presence of *F. circinatum* was only confirmed in the seedlings that had been inoculated with this pathogen, and it was not detected in the control or the other groups of seedlings.

Two-month-old infected pine seedlings showed typical symptoms of damping-off, with up to 32% mortality (depending on the provenance considered) in the two months after inoculation. Survival analysis demonstrated the susceptibility of *P. sylvestris* to *F. circinatum* over a period of four months (Figure 1).

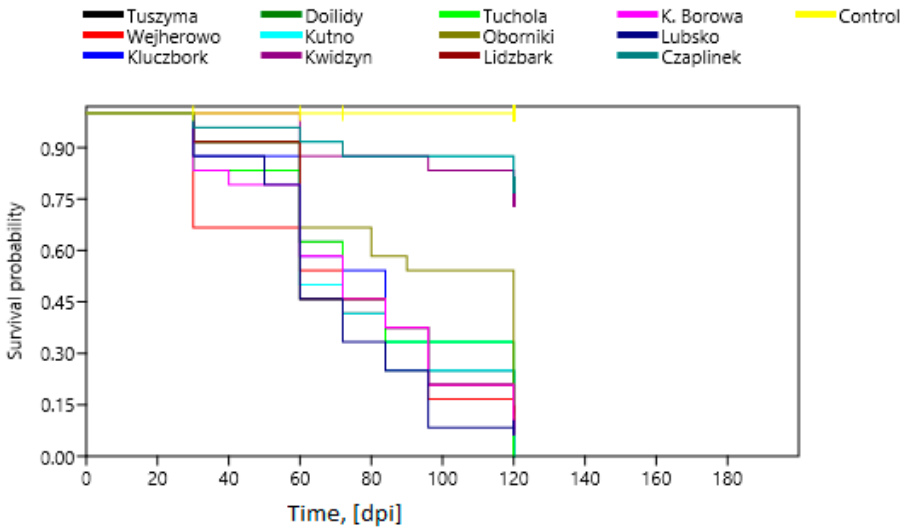

Figure 1. Plot of survival probability determined using the Kaplan-Meier test estimates the survival function for 12 provenances of *Pinus sylvestris* seedlings infected with *Fusarium circinatum*.

The final inspection of the 12 provenances, four months after inoculation, generally revealed severe plant mortality (up to 100%) in ten provenances, while two provenances (Kwidzyń and Czaplinek) displayed a high survival rate (viability rating from 81.8%–86.3%) (Figure 1). Cumulative proportions of survival, compared using the Kaplan–Meier estimate, differed significantly between provenances ($F = 4.4445$, p-value = 0.0027). Survival of control seedlings was 100% at the end of the experiment and

was significantly higher than the mean survival of all the inoculated groups (Figure 1). The mortality rate of seedlings of the two provenances with the highest survival rates was significantly higher than in the control group ($p = 0.000179$) and the other ten provenances ($p = 0.00002$).

4. Discussion

4.1. Preliminary Assessment of Pathogenicity of Fusarium Circinatum to Polish Pine Provenances

Previous studies carried out in Europe, the US and Asia have shown considerable differences in the susceptibility of different pine species to *F. circinatum* [30–32]. Most studies have reported that seedlings and older plants of *P. sylvestris* are moderately to highly susceptible to the pathogen [33–35]. Some research conducted in infested nurseries [7] revealed that the pathogen was only isolated from *P. radiata* and *P. pinaster*, while *Pinus nigra* J.F. Arnold, *P. sylvestris* and *Pseudotsuga menziesii* seedlings did not show any symptoms of pitch canker. The results of the present study indicated that if *F. circinatum* appears in Poland, it will be an important pathogen of *P. sylvestris* and could affect and kill pine seedlings within a short period of time. Studies of this type generally rely on inoculation data from greenhouse or field results for the rapid, accurate assessment of the risk the pathogen poses a serious threat to plants.

Previous studies [36,37] on the resistance of Monterey pine (*Pinus radiata*) and bishop pine (*Pinus muricata* D.Don) to *F. circinatum* showed significant differences in the length of lesions between trees. In the present study, inoculation of one-year-old seedlings in greenhouse trials led to formation of lesions of different lengths. After two months, 30% of seedlings formed lesions shorter than 5 mm, and 40% of symptomatic seedlings had necrotic lesions longer than 5 mm, and the longest lesions were observed in the dead seedlings (data not shown). The inoculations were performed under controlled conditions in a greenhouse to prevent the influence of stress factors such as drought/flood, insect pests or other pathogens on the results. Seedlings with the smallest lesions were considered more or less tolerant to the pathogen at this stage. However, some recent studies have revealed that *F. circinatum* may occur in a latent form [38], suggesting that PPC symptoms may develop up until death of the seedlings.

During the four-month trial period, only 10% of the trees that survived had lesions shorter than 5 mm. The scores for seedling tolerance were therefore similar during the initial period after inoculation. Four months after inoculation, the mean lesion length did not differ significantly between individual seedlings. The comparison showed that *F. circinatum* caused significantly longer necrotic lesions than the inoculated seedlings in the control group ($p < 0.05$).

By contrast, during the greenhouse trial, significant differences were observed in the susceptibility of two-month-old seedlings of 12 Polish provenances of Scots pine to *F. circinatum*. The absence of symptoms on the seedlings of the Kwidzyń and Czaplinek provenances during the entire study period (compared with the other ten provenances, which were severely affected) indicates that selection for genetic resistance to *F. circinatum* may be conducted to improve resistance to pine pitch canker [39]. Assessment of the development of the symptoms in the Polish provenances of *P. sylvestris* show that all except the Kwidzyń and Czaplinek provenances are highly susceptible to pitch canker. This may be because the plants were too young to develop effective physiological mechanisms of plant resistance. Genetic variability in populations of *P. sylvestris* growing in eight locations Poland has been described on the basis of cone morphology [40]. The populations were found to form two geographic groups, in the northeastern and southwestern areas [40]. However, no differences in the genetic variability of Polish provenances of Scots pine have been identified in relation to several genetic markers [41]. The variation in the susceptibility to pitch canker observed in the present study may drive the implementation of genetic breeding programs aimed at management of this disease in the future. However, the significant differences in the tolerance of two-month-old seedlings of 12 Polish provenances to *F. circinatum* and the high viability of seedlings observed in the four months after inoculation suggest that further studies should first be conducted to obtain conclusive results.

Survival analysis of live seedlings was similar to that conducted with live trees and may distort the results [42], with delayed mortality of plants being interpreted as tolerance to the pathogen. Seedlings that survived after inoculation with *F. circinatum* were excluded from the Kaplan–Meier estimate as the time to death was not known [42]. However, some studies demonstrate that infection by *F. circinatum* can occur during the latent phase. Moreover, latent infection influences the susceptibility of Monterey pine seedlings to pitch canker [43]; further observation of inoculated seedlings over a longer period is planned for a more detailed evaluation of the susceptibility of seedlings to pitch canker.

Nevertheless, the high level of susceptibility of *P. sylvestris* indicates a potential risk to this important tree species in the event that *F. circinatum* is introduced into Poland, at least in nursery facilities where *F. circinatum* could spread worldwide.

4.2. Virulence of Other Fusarium Species

Damping off is the most common and serious disease affecting seedlings in forest nurseries in many countries. Many species of *Fusarium* are found even in very poor and degraded soil, resulting in financial losses, mostly in nurseries and forest plantations. *Fusarium* spp. are commonly found to affect the roots of coniferous seedlings, causing damping-off and wilt in pine seedlings [44,45]. Our findings demonstrate that all of the *Fusarium* species tested successfully infected pine seedlings. All six *Fusarium* species caused damping-off disease or weakening in 15-day-old pine seedlings. *F. oxysporum* is the most common pathogen in Polish forest nurseries and was found to be the most virulent to seedlings. *F. tricinctum* displayed lower virulence than *F. oxysporum* but much higher virulence than the other *Fusarium* species. The findings are consistent with those of other studies Polish nurseries where serious problems exist with damping-off rot caused by the *Fusarium* species [17,18]. Apart from *F. circinatum*, the *Fusarium* species tested are better known as pathogens of agricultural crops; *Fusarium poae*, *F. graminearum* (A and D) and *F. culmorum* were particularly less virulent to *P. sylvestris* seedlings than *F. oxysporum* and *F. tricinctum*, which displayed much more variable levels of virulence. These species may be the first potential invaders to transfer from agricultural to forest lands. However, the greenhouse trial and measurement of the mean length of lesions demonstrated that one-year-old seedlings of *P. sylvestris* were not significantly susceptible to the Polish isolates of *Fusarium* spp. tested. No symptoms of damping-off or root necrosis were observed during the 4-month trial period. These results suggest that one-year-old seedlings of *P. sylvestris* appeared to be resistant to *F. oxysporum*, *F. tricinctum*, *F. poae*, *F. graminearum* (A and D) and *F. culmorum*. In a previous study, preliminary assessment of the pathogenicity of pine seedlings in Petri dishes proved to be an effective method of determining the pathogenic capacity of *Fusarium* species prior to greenhouse trials [45]. As expected, the fifteen-day-old pine seedlings were more sensitive to the most of *Fusarium* species tested than one-year-old pine seedlings, in which root growth inhibition was less important.

The study findings demonstrated that *F. oxysporum* may act as a serious root rot pathogen in pine seedlings, while the virulence of *F. tricinctum* is moderate or low. At the same time, all the *Fusarium* species tested did not appear to pose an important risk to one-year-old pine seedlings, which is good news for forest nursery owners. These findings may be of interest to private and state owners of agricultural land destined for afforestation, as such land may be colonized by infectious seed-borne *Fusarium*, originating from agricultural crops. The study findings also show that the risk of pine seedlings planted in former farmland in Poland being infected with *Fusarium* spp. is rather low, despite the presence in the soil of *Fusarium* species known to affect agricultural crops.

The study findings indicate that Polish provenances of Scots pine seedlings are susceptible to damping-off caused by *F. circinatum* and an invasion of this pathogen represents a serious threat to Polish forestry.

5. Conclusions

The results of the present study indicate that if the fungus *F. circinatum* reaches Scots pine in Poland nurseries, it will be very damaging and probably kill pine seedlings quite rapidly. Greenhouse

trials involving inoculation of two-month-old and one-year-old seedlings demonstrated the high susceptibility of Scots pine seedlings to pitch canker disease, although two provenances showed signs of higher tolerance. This illustrates the potential usefulness of investigating the genetic resistance of individual clones to *F. circinatum* (for potential breeding programs) to enable control of this disease if it becomes established in forest ecosystems.

The pine shoot beetle (*Tomicus piniperda*) and other bark beetles are considered potential vectors of *F. circinatum* as they could transmit the pathogen during maturation feeding on the shoots of healthy pine trees [46,47]. As *T. piniperda* is one of the most common bark beetles in Poland, it could increase the potential risk of spread of the pathogen and disease throughout the country.

The correlation between the susceptibility of provenances and individuals to *F. circinatum* and possibility of the pathogen being transferred by bark beetles may require studies in field trees and nursery plants [8,40,47]. However, field research on *F. circinatum* in Poland is restricted by quarantine measures and therefore experiments are generally limited to greenhouses in the Research Centre of Quarantine Organisms. Nonetheless, further studies will be conducted with inoculated and non-inoculated seedlings to determine which genotypes are most resistant to pitch canker by focusing on individual resistance of seedlings.

Moreover, our findings indicate that other species of Fusarium (*F. poae*, *F. graminearum* and *F. culmorum*) are significantly less virulent to *P. sylvestris* seedlings than *F. oxysporum* and *F. tricinctum*. However, only *F. oxysporum* had moderate virulence, while *F. poae, F. graminearum* and *F. culmorum* proved to be weakly virulent or non-virulent.

In summary, the present study confirms the importance of carrying out susceptibility tests with Polish provenances of Scots pine with the aim of facilitating a breeding program for this tree species, as well as the need to explore the individual resistance of pine genotypes in preparation to deal with the problem of the probable appearance and establishment of *Fusarium circinatum* in Poland.

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References

1. Santini, A.; Ghelardini, L.; De Pace, C.; Desprez-Loustau, M.L.; Capretti, P.; Chandelier, A.; Cech, T.; Chira, D.; Diamandis, S.; Gaitniekis, T.; et al. Biogeographical Patterns and Determinants of Invasion by Forest Pathogens in Europe. *New Phytol.* **2013**, *197*, 238–250. [CrossRef] [PubMed]
2. Ganley, R.J.; Watt, M.S.; Kriticos, D.J.; Hopkins, A.J.M.; Manning, L.K. Increased Risk of Pitch Canker to Australasia under Climate Change. *Australas. Plant Pathol.* **2011**, *40*, 228–237. [CrossRef]
3. Watt, M.S.; Ganley, R.J.; Kriticos, D.J.; Manning, L.K. Dothistroma Needle Blight and Pitch Canker: The Current and Future Potential Distribution of Two Important Diseases of Pinus Species. *Can. J. For. Res.* **2011**, *41*, 412–424. [CrossRef]

4. Gordon, T.R.; Kirkpatrick, S.C.; Aegerter, B.J.; Fisher, A.J.; Storer, A.J.; Wood, D.L. Evidence for the Occurrence of Induced Resistance to Pitch Canker, Caused by *Gibberella circinate* (Anamorph *Fusarium circinatum*), in Populations of *Pinus radiata*. *For. Pathol.* **2011**, *41*, 227–232. [CrossRef]
5. Gordon, T.R. Pitch Canker Disease of Pines. *Phytopathology* **2006**, *96*, 657–659. [CrossRef] [PubMed]
6. European Food Safety Authority (EFSA). Risk Assessment of *Gibberella circinata* for the EU Territory and Identification and Evaluation of Risk Management Options. *EFSA J.* **2010**, *8*, 1620. [CrossRef]
7. Bezos, D.; Martinez-Alvarez, P.; Fernández, M.; Diez, J.J. Epidemiology and Management of Pine Pitch Canker Disease in Europe—A Review. *Balt. For.* **2017**, *23*, 279–293.
8. Landeras, E.; García, P.; Fernández, Y.; Braña, M.; Fernández-Alonso, O.; Méndez-Lodcs, S.; Pérez-Sierra, A.; León, M.; Abad-Campos, P.; Berbegal, M.; et al. Outbreak of Pitch Canker Caused by *Fusarium circinatum* on *Pinus* spp. in Northern Spain. *Plant Dis.* **2005**, *89*, 1015. [CrossRef]
9. Bragança, H.; Diogo, E.; Moniz, F.; Amaro, P. First Report of Pitch Canker on Pines Caused by *Fusarium circinatum* in Portugal. *Plant Dis.* **2009**, *93*, 1079. [CrossRef]
10. Wingfield, M.J.; Hammerbacher, A.; Ganley, R.J.; Steenkamp, E.T.; Gordon, T.R.; Wingfield, B.D.; Coutinho, T.A. Pitch Canker Caused by Fusarium circinatum—A Growing Threat to Pine Plantations and Forests Worldwide. *Australas. Plant Pathol.* **2008**, *37*, 319–334. [CrossRef]
11. European and Mediterranean Plant Protection Organization (EPPO). PQR Database. Paris, France. 2014. Available online: http://www.eppo.int/DATABASES/pqr/pqr.htm (accessed on December 2016).
12. Carlucci, A.; Colatruglio, L.; Frisullo, S. First Report of Pitch Canker Caused by *Fusarium circinatum* on *Pinus halepensis* and *P. pinea* in Apulia (Southern Italy). *Plant Dis.* **2007**, *91*, 1683.
13. EPPO. *Pm 7/91(1): Gibberella circinata. EPPO Bull*; EPPO: Paris, France, 2009; pp. 298–309.
14. Chelkowski, J. (Ed.) *Fusarium: Mycotoxins, Taxonomy, Pathogenicity*; Elsevier: Amsterdam, The Netherlands, 1989; Volume 2, p. 492.
15. Martín-Pinto, P.; Pajares, J.; Díez, J. Pathogenicity of *Fusarium verticillioides* and *Fusarium oxysporum* on *Pinus nigra* Seedlings in Northwest Spain. *For. Pathol.* **2008**, *38*, 78–82. [CrossRef]
16. Anderson, I.C.; Campbell, C.D.; Prosser, J.I. Diversity of Fungi in Organic Soils under a Moorland–Scots Pine (*Pinus sylvestris* L.) Gradient. *Environ. Microbiol.* **2003**, *5*, 1121–1132. [CrossRef] [PubMed]
17. Machón, P.; Pajares, J.A.; Diez, J.J.; Alves-Santos, F.M. Influence of the Ectomycorrhizal Fungus *Laccaria laccata* on Pre-Emergence, Post-Emergence and Late Damping-Off by *Fusarium oxysporum* and *F. verticillioides* on Stone Pine Seedlings. *Symbiosis* **2009**, *49*, 101–109. [CrossRef]
18. Duda, B.; Sierota, Z.H. Survival of Scots Pine Seedlings after Biological and Chemical Control of Damping-off in Plastic Greenhouses. *For. Pathol.* **1987**, *17*, 110–117. [CrossRef]
19. Bełka, M.; Mańka, M. Damping-off of Scots pine (*Pinus sylvestris*) Seedlings in 2004 and 2005 in Nowy Dwór Forest Nursery *versus* Soil Fungi Communities. *Phytopathologia* **2010**, *58*, 63–67.
20. Martínez-Alvarez, P.; Pando, V.; Diez, J.J. Alternative Species to Replace Monterey Pine Plantations Affected by Pitch Canker Caused by *Fusarium circinatum* in Northern Spain. *Plant Pathol.* **2014**, *63*, 1086–1094. [CrossRef]
21. James, R.L.; Dumroese, R.K.; Wenny, D.L. *Fusarium proliferaturm* is a Common, Aggressive Pathogen of Container-Grown Conifer Seedling. *Phytopathology* **1995**, *85*, 1129.
22. Stewart, J.E.; Abdo, Z.; Dumroese, R.K.; Klopfenstein, N.B.; Kim, M.S. Virulence of *Fusarium oxysporum* and *F. Commune* to Douglas-Fir (*Pseudotsuga menziesii*) Seedlings. *For. Pathol.* **2012**, *42*, 220–228. [CrossRef]
23. Schweigkofler, W.; O'Donnell, K.; Garbelotto, M. Detection and Quantification of Airborne Conidia of Fusarium circinatum, the Causal Agent of Pine Pitch Canker, from Two California Sites by Using a Real-Time PCR approach Combined with a Simple Spore Trapping Method. *Appl. Environ. Microbiol.* **2004**, *70*, 3512–3520. [CrossRef] [PubMed]
24. Glass, N.L.; Donaldson, G.C. Development of Primer Sets Designed for Use with the PCR to Amplify Conserved Genes from Filamentous Ascomycetes. *Appl. Environ. Microbiol.* **1995**, *61*, 1323–1330. [PubMed]
25. Kristensen, R.; Torp, M.; Kosiak, B.; Holst-Jensen, A. Phylogeny and Toxigenic Potential is Correlated in Fusarium Species as Revealed by Partial Translation Elongation Factor 1 Alpha Gene Sequences. *Mycol. Res.* **2005**, *109*, 173–186. [CrossRef] [PubMed]
26. Gardes, M.; Bruns, T.D. Its Primers with Enhanced Specificity for Basidiomycetes—Application to the Identification of Mycorrhizae and Rusts. *Mol. Ecol.* **1995**, *2*, 113–118. [CrossRef]

27. White, T.J.; Bruns, T.; Lee, S.; Taylor, J. Amplification and Direct Sequencing of Fungal Ribosomal RNA Genes for Phylogenetics. In *PCR Protocols: A Guide to Method and Applications*; Innis, M.A., Gelfand, D.H., Sninsky, J.J., White, T.J., Eds.; Academic Press: New York, NY, USA, 1990; pp. 315–322.
28. Altschul, S.F.; Gish, W.; Miiller, W.; Myers, E.W.; Lipman, D.J. Basic Local Alignment Search Tool. *J. Mol. Biol.* **1997**, *215*, 403–410. [CrossRef]
29. Therneau, T. A Package for Survival Analysis in S. R Package Version 2.41-3. Available online: https://CRAN.R-project.org/package=survival (accessed on 18 December 2017).
30. Wingfield, M.J.; Jacobs, A.; Coutinho, T.A.; Ahumada, R.; Wingfield, B.D. First Report of the Pitch Canker Fungus, *Fusarium circinatum*, on Pines in Chile. *Plant Pathol.* **2002**, *51*, 397. [CrossRef]
31. Hodge, G.R.; Dvorak, W.S. Variation in Pitch Canker Resistance Among Provenances of *Pinus patula* and *Pinus tecunumanii* from Mexico and Central America. *New For.* **2007**, *33*, 193–206. [CrossRef]
32. Enebak, S.A.; Stanosz, G.R. Responses of Conifer Species of the Great Lakes Region of North America to Inoculation with the Pitch Canker Pathogen *Fusarium circinatum*. *For. Pathol.* **2003**, *33*, 333–338. [CrossRef]
33. Iturritxa, E.; Ganley, R.J.; Raposo, R.; García-Serna, I.; Mesanza, N.; Kirkpatrick, S.C.; Gordon, T.R. Resistance Levels of Spanish Conifers Against *Fusarium circinatum* and *Diplodia pinea*. *For. Pathol.* **2013**, *43*, 488–495. [CrossRef]
34. Pérez-Sierra, A.; Landeras, E.; León, M.; Berbegal, M.; García-Jiménez, J.; Armengol, J. Characterization of *Fusarium circinatum* from *Pinus* spp. in Northern Spain. *Mycol. Res.* **2007**, *111*, 832–839. [CrossRef] [PubMed]
35. Martín-García, J.; Lukačevičová, A.; Flores-Pacheco, J.A.; Diez, J.J.; Dvořák, M. Evaluation of the Susceptibility of Several Czech Conifer Provenances to *Fusarium circinatum*. *Forests* **2018**, *9*, 72.
36. Storer, A.J.; Gordon, T.R.; Clark, S.L. Association of the Pitch Canker Fungus, Fusarium Subglutinans f. sp. pini with Monterey Pine Seeds and Seedlings in California. *Plant Pathol.* **1998**, *47*, 649–656. [CrossRef]
37. Schmale, D.G.; Gordon, T.R. Variation in Susceptibility to Pitch Canker Disease, caused by *Fusarium circinatum*, in Native Stands of *Pinus muricata*. *Plant Pathol.* **2003**, *52*, 720–725. [CrossRef]
38. Swett, C.L.; Kirkpatrick, S.C.; Gordon, T.R. Evidence for a Hemibiotrophic Association of the Pitch Canker Pathogen *Fusarium circinatum* with *Pinus radiata*. *Plant Dis.* **2016**, *100*, 79–84. [CrossRef]
39. Martín-García, J.; Paraschiv, M.; Flores-Pacheco, J.A.; Chira, D.; Diez, J.J.; Fernández, M. Susceptibility of Several Northeastern Conifers to *Fusarium circinatum* and Strategies for Biocontrol. *Forests* **2017**, *8*, 318.
40. Nowakowska, J.A.; Zachara, T.; Konecka, A. Genetic Variability of Scots pine (*Pinus sylvestris* L.) and Norway Spruce (*Picea abies* L. Karst.) Natural Regeneration Compared with Their Maternal Stands. *For. Res. Pap.* **2014**, *75*, 47–54. [CrossRef]
41. Bobowicz, M.A.; Korczyk, A.F. Variability of Eight Polish Populations of *Pinus sylvestris* L. Expressed in Traits of Cones. *Acta Soc. Bot. Pol.* **1994**, *63*, 191–198. [CrossRef]
42. Vivas, M.; Zas, R.; Solla, A. Screening of Maritime Pine (*Pinus pinaster*) for Resistance to *Fusarium circinatum*, the Causal Agent of Pitch Canker Disease. *Forestry* **2011**, *85*, 185–192. [CrossRef]
43. Swett, C.L.; Gordon, T.R. Latent Infection by *Fusarium circinatum* Influences Susceptibility of Monterey Pine Seedlings to Pitch Canker. In Proceedings of the Fourth International Workshop on the Genetics of Host-Parasite Interactions in Forestry: Disease and Insect Resistance in Forest Trees, Eugene, OR, USA, 31 July–5 August 2011; pp. 159–161.
44. Kwaśna, H.; Bateman, G.L. Microbial Communities in Roots of *Pinus sylvestris* Seedlings with Damping-Off Symptoms in Two Forest Nurseries as Determined by ITS1/2 rDNA Sequencing. *For. Pathol.* **2009**, *39*, 239–248. [CrossRef]
45. Lazreg, F.; Belabid, L.; Sanchez, J.; Gallego, E.; Bayaa, B. Pathogenicity of *Fusarium* spp. Associated with Diseases of Aleppo-Pine Seedlings in Algerian Forest Nurseries. *J. For. Sci.* **2014**, *60*, 115–120. [CrossRef]
46. Romón, P.; Iturrondobeitia, J.C.; Gibson, K.; Lindgren, B.S.; Goldarazena, A. Quantitative Association of Bark Beetles with Pitch Canker Fungus and Effects of Verbenone on their Semiochemical Communication in Monterey Pine Forests in Northern Spain. *Environ. Entomol.* **2007**, *36*, 743–750. [CrossRef] [PubMed]
47. Bezos, D.; Martínez-Álvarez, P.; Diez, J.J. The Pine Shoot Beetle *Tomicus piniperda* as a Plausible Vector of *Fusarium circinatum* in Northern Spain. *Ann. For. Sci.* **2015**, *72*, 1079. [CrossRef]